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ORIGINAL INVESTIGATION

Reduced astrocyte density underlying brain volume reduction in activity-based anorexia rats

Linda Frintrop^{a*}, Johanna Liesbrock^{a,b*}, Lisa Paulukat^{a,b}, Sonja Johann^a, Martien J. Kas^{c,d}, Rene Tolba^e, Nicole Heussen^f, Joseph Neulen^g, Kerstin Konrad^b, Beate Herpertz-Dahlmann^b, Cordian Beyer^a and Jochen Seitz^b

^aInstitute of Neuroanatomy, University Hospital, RWTH Aachen University, Aachen, Germany; ^bDepartment of Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy, University Hospital, RWTH Aachen University, Aachen, Germany; ^cDepartment of Translational Neuroscience, Brain Center Rudolf Magnus, University Medical Center Utrecht, Utrecht, The Netherlands; ^dGroningen Institute for Evolutionary Life Sciences, University of Groningen, the Netherlands; ^eInstitute for Laboratory Animal Science and Experimental Surgery, University Hospital, RWTH Aachen University, Aachen, Germany; ^fDepartment of Medical Statistics, University Hospital Aachen, RWTH Aachen University, Aachen, Germany; ^gDepartment of Gynecological Endocrinology and Reproductive Medicine, University Hospital, RWTH Aachen University, Aachen, Germany

ABSTRACT

Objectives: Severe grey and white matter volume reductions were found in patients with anorexia nervosa (AN) that were linked to neuropsychological deficits while their underlying pathophysiology remains unclear. For the first time, we analysed the cellular basis of brain volume changes in an animal model (activity-based anorexia, ABA).

Methods: Female rats had 24 h/day running wheel access and received reduced food intake until a 25% weight reduction was reached and maintained for 2 weeks.

Results: In ABA rats, the volumes of the cerebral cortex and corpus callosum were significantly reduced compared to controls by 6% and 9%, respectively. The number of GFAP-positive astrocytes in these regions decreased by 39% and 23%, total astrocyte-covered area by 83% and 63%. In neurons no changes were observed. The findings were complemented by a 60% and 49% reduction in astrocyte (GFAP) mRNA expression.

Conclusions: Volumetric brain changes in ABA animals mirror those in human AN patients. These alterations are associated with a reduction of GFAP-positive astrocytes as well as GFAP expression. Reduced astrocyte functioning could help explain neuronal dysfunctions leading to symptoms of rigidity and impaired learning. Astrocyte loss could constitute a new research target for understanding and treating semi-starvation and AN.

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Introduction

Anorexia nervosa (AN) is the third most common chronic disease in adolescence and young adulthood (Gonzalez et al. 2007). It is a psychiatric disorder characterised by insufficient food intake, severe body weight loss, fear of weight gain and a disturbed representation of one's own body (American Psychiatric Association 2013). It has a strong genetic background but also environmental factors like thin ideal and dieting play an important role (Hinney et al. 2016; Stice et al. 2016). The onset occurs primarily during adolescence in young women and 30–80% of the patients show a characteristic physical hyperactivity (Herpertz-Dahlmann 2015). Effective treatments are limited (Zipfel et al. 2015) and the mortality rate is the highest

of all psychiatric disorders (Fichter and Quadflieg 2016).

Striking brain volume deficits are well-documented in patients with AN. In a recent meta-analysis including 216 patients their grey matter showed a 4.6% reduction in comparison to healthy controls. This effect was even more pronounced in adolescents with AN (8.4%). White matter was reduced by 2.7%, also more pronounced for adolescents (4.0%) (Seitz et al. 2016). After weight restoration, grey and white matter alterations were largely reversed (Mainz et al. 2012; King et al. 2015). However, it remained unclear whether it was a 'restitutio ad integrum' or whether a 'scar' remained in the brain of former patients (Seitz et al. 2014). Grey matter volume loss has been

CONTACT Linda Frintrop ✉ lfrintrop@ukaachen.de 📧 Master of Science Biology, Institute for Neuroanatomy, RWTH University Aachen, Wendlingweg 2, 52074 Aachen, Germany

*Linda Frintrop and Johanna Liesbrock contributed equally to the work.

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associated with a greater drive for thinness (Joos et al. 2011), reduced visuo-spatial capacities (Castro-Fornieles et al. 2010) and impaired logical thinking (McCormick et al. 2008). Notably, the decrease in white matter volume in acutely ill patients with AN and failure to increase grey matter upon weight restoration are associated with a negative prognosis and an impaired weight recovery at 1-year follow-up (McCormick et al. 2008; McCormick et al. 2009; Seitz et al. 2015). Additional studies have shown alterations in gyrification level of the brain that were associated with clinical outcome (Favaro et al. 2015) and microstructural alterations of white matter, including the corpus callosum, that were associated with the severity of starvation and the duration of the eating disorder (Kazlouski et al. 2011; Frieling et al. 2012; Yau et al. 2013; Nagahara et al. 2014; Vogel et al. 2016).

The underlying cellular mechanisms of this volume reduction are largely unclear as systematic data are lacking. Human post-mortem case studies in three chronically ill girls with AN seemed to indicate that neurons showed signs of cellular degeneration, long and thin dendrites and an altered spine morphology (Martin 1958; Neumärker et al. 1997).

The most commonly used animal model that mimics the behavioural and physiological aspects of AN is the activity-based anorexia (ABA) rat model. When given access to running wheels, most food-restricted rodents start to run voluntarily despite utilising even more energy and exhibit other core symptoms of AN, including amenorrhoea (Watanabe et al. 1992; Paulukat et al. 2016), hypothermia (Hillebrand et al. 2005) and hypoleptinemia (Pardo et al. 2010). Previous findings in ABA rats indicate that also glia could be affected, including decreased cell proliferation in the dentate gyrus, the surrounding dorsal hippocampus and the corpus callosum (Barbarich-Marsteller et al. 2013). These effects were not prominent in regions with known neurogenesis, such as the subgranular zone of the dentate gyrus, leading the authors of that paper to suspect a primary effect on gliogenesis. Furthermore, Reyes-Haro et al. (2015) showed that the astrocyte count was slightly reduced in a different animal model for AN, which is based on acute dehydration, in a subregion of the corpus callosum. However, neither paper combined volumetric and cellular measurements.

To date, the starvation-induced effects on brain volume reduction and their cellular underpinnings have not been systematically analysed in animal models of AN. As a disturbed development of the brain during adolescence with subsequent scars might be one

reason for the chronicity of AN and a high comorbidity with other psychiatric disorders (Mainz et al. 2012), a distinct knowledge about the effect of starvation on the brain is urgently needed. This knowledge would not only be relevant for the development of new interventions of AN but also have important implications for improving our understanding of the neurobiological consequences of semi-starvation due to undernutrition or secondary to chronic medical conditions associated with severe weight loss. The first aim of this study was to investigate whether the starvation-induced reduction of cerebral cortex and corpus callosum volumes found in patients with AN could also occur in female adolescent ABA rats. Our second aim was to further elucidate the pathophysiological and cellular mechanisms underlying this volume loss. We thus analysed alterations in neuron and astrocyte numbers, cell areas and mRNA expression in chronically starved ABA rats in comparison with normally developing control animals.

Materials and methods

Subjects

Forty-one adolescent 4-week-old female Wistar rats (Charles River, Sulzfeld, Germany) arrived at the laboratory with an average weight of 87.5 g (SD 15.3). They were individually housed in Type IV 1820 cm² cages (Polysulfone, Tecniplast GmbH, Germany) under a 12-h light/dark cycle (lights on at 07:00 h) with *ad libitum* access to water and all rats had 24 h/day running wheel access. The facility is specific pathogen free according to the FELASA Guidelines and certified according to DIN ISO 9001/2008.

Study design

The ABA paradigm of self-starvation (Routtenberg & Kuznesof 1967; Kas et al. 2003; Gutierrez 2013) was altered to allow for testing of chronic starvation. A schematic summary is shown in Paulukat et al. (2016). In brief, the rodents were allowed a 10-day acclimatisation phase in their single running wheel cages with food *ad libitum*. After habituation, animals were randomly assigned to experimental groups. At first, 11 animals in the ABA group received 40% of their baseline daily food intake until they lost 25% of their weight. For 2 additional weeks they received adjusted daily food intake to the needs of the individual animal to hold a stable weight (ABA_chronic). This was achieved by weighing the animals every day and adapting their food for the next 24 h to between 60% and 80% of

their baseline daily food intake, depending on the difference to the target weight. Food consumption, body weight, running wheel activity (RWA; analysed with tachometers, BC 5.12, Sigma, Germany) and menstrual cycles were measured daily at 12.00 h. Twelve control animals were housed under the same terms but fed *ad libitum* and sacrificed after the same number of days as the experimental group (Controls_chronic: $n = 12$). For an additional analysis of short-term starvation, and to determine whether the duration of starvation was an important factor, nine animals were treated in the same way as described above, but they were sacrificed directly after reaching their target weight (ABA_acute) and compared with a separate control group with nine rats sacrificed after the respective number of days (Controls_acute).

All animal experiments were approved by the Governmental Animal Care and Use Committee of the Ministry for Nature, Environment and Consumer Protection of the State of North Rhine-Westphalia and carried out in agreement with the German Animal Protection Law and European regulations (Guideline 86/609/EECDirective 2010/63).

Volume measurement

After anaesthesia with isoflurane (Forene, 100%, v/v, B506, Abbott) and transcardial perfusion with a 150-mL artificial cerebrospinal fluid solution, the brains were rapidly removed from the skull and divided into two hemispheres at the midsagittal line. The right cerebral hemispheres were used for cryo-sectioning and volume analysis, the left hemispheres were used for a direct preparation of the somatosensory area and the corpus callosum following mRNA isolation. The right brain halves were post-fixed with a 3.7% paraformaldehyde solution (pH 7.4) for 2 days, rinsed in tap water and then cryo-protected by immersion overnight in 10% and 30% sucrose in phosphate-buffered saline at 4 °C. Afterwards, the hemispheres were embedded in optimal cutting temperature medium and stored at -80 °C until further processing.

The entire right hemisphere of each animal was cut frontally in a series of 100- μ m sections on a cryostat (Leica CM 3050S, Nussloch, Germany) and then thaw-mounted on gelatinised glass slides. Every second slice was stained with haematoxylin-eosin. Slides were dried overnight, incubated for 30 min in 0.1% haematoxylin solution (Merck 517282, Darmstadt, Germany), then flushed for 10 min with tap water and incubated for 10 min in 0.2% eosin solution (Merck 115935, Darmstadt, Germany). In addition, they were dehydrated in an ascending sequence

with ethanol and afterwards with xylol. Finally, the slides were cover-slipped with DePeX (Serva, Heidelberg, Germany).

Stained slices were digitalised and the areas of interest (cerebral cortex and corpus callosum) of every second slice were determined manually by tracing with ImageJ software (1.48v, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA), using the Cavalieri method by an observer blinded to the experimental treatment groups. The areas were multiplied by the distance between the histological sections and summed to yield the total volume. The volume was analysed for the cerebral cortex from Bregma 5.20 to -9.80 and for the corpus callosum from 3.70 to -8.00 using the rat brain atlas from Paxinos and Watson, encompassing both compartments completely. One brain hemisphere of an ABA rat had to be excluded because the halves were accidentally switched.

Immunohistochemistry

A series of intermittent 20 μ m sections were made with a cryostat at Bregma -2.30. Two sections per animal were chosen from this Bregma location for staining. Sections were exposed overnight at 37 °C, then incubated with 5% goat or horse serum (Sigma, Munich, Germany) for 1 h and exposed overnight at 4 °C to the following primary antibodies: goat anti-glial fibrillary acid protein (GFAP) polyclonal antibody (astroglia, 1:750; catalogue number: sc-6170, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-microtubule-associated protein 2 (Map2) monoclonal antibody (neurons, 1:1500; catalogue number: D5G1, Cell Signalling Technology, USA). After washing, we unmasked the sections by citrate (pH 6.0) and all sections were then treated with H₂O₂/methanol (0.3% vol., Roth, Karlsruhe, Germany). Subsequently, the sections were incubated with the appropriate secondary antibodies followed by the ABC complex (Vector Laboratories, Burlingame, CA, USA). Afterwards, a Vectastain-DAB Kit (Vector Laboratories, Burlingame, CA, USA) was used and sections were counterstained with haematoxylin, dehydrated in graded alcohol and mounted. Slices of sufficient quality could be obtained for ten animals of the ABA_chronic and eleven animals of the Control_chronic group.

To show the specificity of cellular changes for GFAP-positive astrocytes, additional mouse anti-adenomatous polyposis coli (APC) monoclonal antibody (1:500; catalogue number: OP80, Calbiochem, Germany) was used to stain and count oligodendrocytes in slices at Bregma 4.16 accordingly.

Cell parameter quantification

Regions of interest were digitally recorded using the Leica DM 6000 microscope (Leica microsystems, Bensheim, Germany). The numbers of GFAP-, Map2- and APC-positive cells were analysed by manual counting with ImageJ 3 software (1.48v, Wayne Rasband, National Institutes of Health) by two observers blinded to the groups, and the results were averaged and expressed as cells/mm². Only cells with a visible nucleus were counted. Immunoreactive areas of GFAP and Map2 cells were determined with ImageJ software by quantifying the GFAP and Map2 signalling as the area in percent. For the corpus callosum analysis, recordings from three different regions (medial, sub cingulum and lateral) were averaged. Also three different areas (retrosplenial granular cortex, primary motor cortex and primary somatosensory cortex) were averaged for the cerebral cortex analysis.

Reverse transcriptase (RT) and real-time polymerase chain reaction (rtPCR)

The mRNA of cerebral cortex and corpus callosum samples were isolated using PeqGold RNA Trifast (Peqlab, Germany). Afterwards, the samples were reverse transcribed in complementary DNA with Invitrogen M-MLV RT-kit and random hexanucleotide primers (Invitrogen, Germany; primer sequences: *CycA* sense: 5'-GGC AAA TGC TGG ACC AAA CAC; *CycA*

antisense: 5'-TTA GAG TTG TCC ACA GTC GGG AGA TG; *GFAP* sense: 5'-AGA AAA CCG CAT CAC CAT T; *GFAP* antisense: 5'-GCA CAC CTC ACA TCA CAT CC; *Map2* sense: 5'-TCG AAA TGC CCG TGG AAT CA; *Map2* antisense: 5'-TGG AAG AAG ACA GGG GCA AAG). The relative expression was measured by calculating the ratio between the gene of interest and the reference gene *cylophilinA* (*cycA*) by the $\Delta\Delta C_t$ -method using the qBase plus software (qBase Biogazelle, Belgium). Changes in gene levels of interest were graphically illustrated by the fold change relative to the control group, with controls set to 100%.

Statistical analysis

The data of all continuous outcomes were described by means and corresponding standard deviations (SD) in each subgroup of acute or chronic starvation of ABA and control animals. Primary outcome was the cerebral cortex volume, while secondary outcomes were the corpus callosum volume, the cell parameters and the mRNA levels. Comparisons between ABA and control animals in acute or chronic starvation were performed by two-sided *t*-tests with a significance level of 5%. Results were reported as *P* values with corresponding degrees of freedom (*t*(df)), values of the test statistic (*t*) and effect sizes (Cohen's *d*). No adjustments for multiple testing were carried out due to the exploratory nature of this study. All analyses were conducted using SPSS version 20 for Windows (IBM,

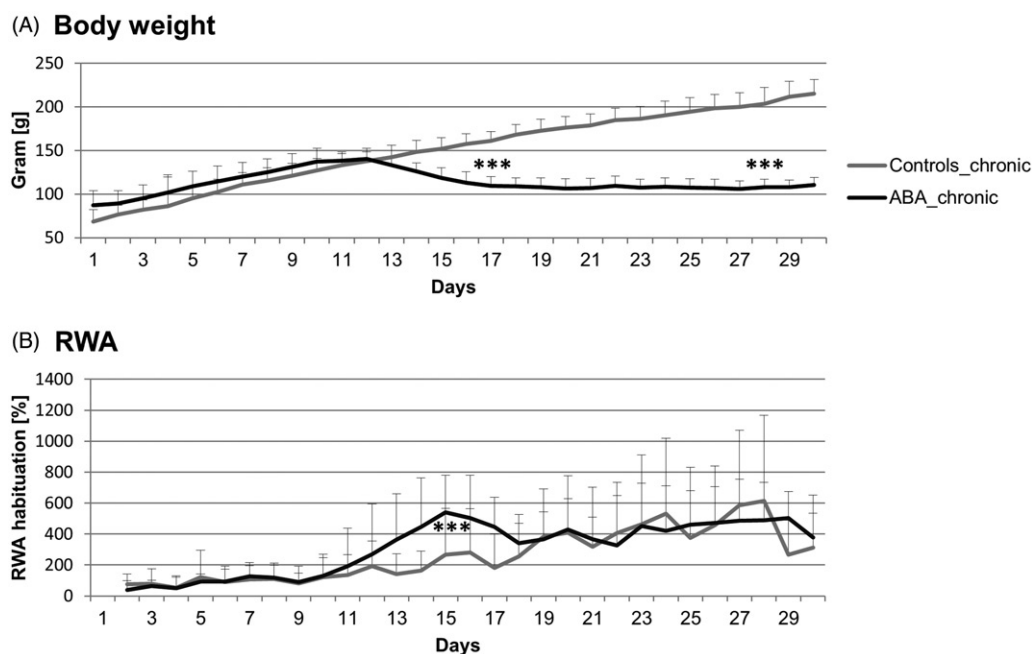


Figure 1. Standardised weight (A) and normalised running wheel activity (RWA, B) in the chronic ABA model as well as in controls. The RWA is normalised to the acclimatisation phase as 100%. (A and B) Two-way ANOVA. *** $P \leq 0.001$. Modified from (Paulukat et al. 2016).

Table 1. Overview of means, standard deviation, *P* values, *t*(df), *t* and Cohen's *d* of the brain volumes, cell count, surface and mRNA results.

	Mean_ABA	SD_ABA	Mean_Controls	SD_Controls	<i>t</i> (df)	<i>t</i>	Cohen's <i>d</i>	<i>P</i> values
Volume_cerebral cortex (mm ³)	219.52	13.76	232.7	14.4	19	2.53	0.93	0.046
Volume_corpus callosum (mm ³)	23.77	2.03	26.15	2.25	20	2.58	1.11	0.02
Number of GFAP-positive astrocytes, cerebral cortex (/mm ³)	37.07	13.31	61.02	21.48	19	3.03	1.32	0.007
Number of GFAP-positive astrocytes, corpus callosum (/mm ³)	71.95	13.83	93.31	11.29	19	3.89	1.70	0.001
GFAP-positive area, cerebral cortex (%)	1.01	0.90	5.8	4.25	18	3.49	1.53	0.002
GFAP-positive area, corpus callosum (%)	1.09	0.54	3.14	1.43	19	4.26	1.86	0.0004
Number of Map2-positive neurons, cerebral cortex (/mm ³)	329.22	119.56	327.53	73.27	16	0.04	0.02	0.971
Map2-positive surface area, cerebral cortex (%)	6.04	6.62	6.48	4.49	13	0.14	0.07	0.89
GFAP mRNA expression, cerebral cortex	0.40	0.12	1.00	0.35	21	5.39	2.25	<0.001
GFAP mRNA expression, corpus callosum	0.49	0.11	1.00	0.25	15	5.56	2.70	<0.001
Map2 mRNA expression, cerebral cortex	0.96	0.33	1.00	0.43	16	0.21	0.10	0.837
Map2 mRNA expression, corpus callosum	1.14	0.27	1.00	0.26	13	1.02	0.53	0.323

Chicago, IL, USA). We also analysed potential correlations between standardised RWA (running wheel activity) and brain volumes using Pearson correlations.

Results

The standardised body weight and normalised RWA of the ABA model and controls are shown in [Figure 1](#). The normalised RWA of ABA rats during the acute starvation phase was significantly higher compared to that of controls (ABA_chronic: 456.66%; Controls_chronic: 204.19%; $P=0.004$), thus showing that the ABA model worked. In the weight-holding phase, however, the RWA of the controls further increased potentially due to longer continuing habituation of the control rats to the running wheel. Interestingly, the increased RWA of the ABA animals stabilised on the high level without further increase, potentially preventing complete over-exertion of the animals. Thus, the RWA of both groups approximated during the weight-holding phase so that it was no longer significantly different between controls and ABA animals (ABA_chronic: 442.36%; Controls_chronic: 431.46%; $P=0.922$). RWA did not correlate with brain volumes (see Supplementary Table 1, available online). In both brain areas, we observed a significant volume reduction. Regarding the volume of the cerebral cortex, there was an approximately 6% decrease in ABA_chronic rats compared to control animals. In the corpus callosum, we found an around 9% volume reduction in ABA_chronic animals (see [Table 1](#) and [Figure 2](#)). To attribute these morphological changes to distinct cell types, we analysed the number of immunoreactive cells and the area covered by these cells. The number of GFAP-positive astrocytes in the cerebral cortex and corpus callosum of chronic ABA rats was significantly reduced in comparison to controls ([Table 1](#) and [Figure 3](#)). The GFAP-positive area was also significantly decreased compared to controls in both

analysed brain regions. The number of Map2-positive neurons in the cerebral cortex of chronically starved rats did not change in comparison to the control group. Similarly, the cell surface area of these neuronal structures in the cerebral cortex of ABA animals was not significantly modified ([Table 1](#) and [Figure 3](#)).

To substantiate the findings of reduced astrocyte numbers and volume, we analysed GFAP expression. In the cerebral cortex, GFAP mRNA expression in ABA rats was downregulated by 60% compared to controls. Furthermore, GFAP mRNA levels in ABA_chronic rats were diminished by approximately 51% in the corpus callosum when compared to the control group ([Table 1](#) and [Figure 4](#)). In both analysed brain regions, Map2 mRNA expression was unchanged.

In addition, we counted the APC-positive oligodendrocytes in the cerebral cortex and corpus callosum to check for cell specificity. There was no alteration in oligodendrocyte numbers in ABA_chronic rats compared to the control group ([Figure 5](#)).

To determine, whether the observed changes were dependent on the duration of starvation, we also analysed ABA_acute rats. In contrast to chronically starved animals, the numbers of GFAP-positive astrocytes and cell surface area in these animals showed no significant alterations compared to controls ([Figure 6](#)).

Discussion

Our study shows reduced cerebral cortex and corpus callosum volumes in the chronic ABA model that parallel the findings in human patients with AN. For the first time, we demonstrate that this volume loss is associated with a reduced number and immunoreactive surface area of GFAP-positive astrocytes but not neurons or oligodendrocytes in both brain regions. This GFAP-positive astrocyte reduction appears to be specific to the chronic starvation condition and was not found after acute starvation.

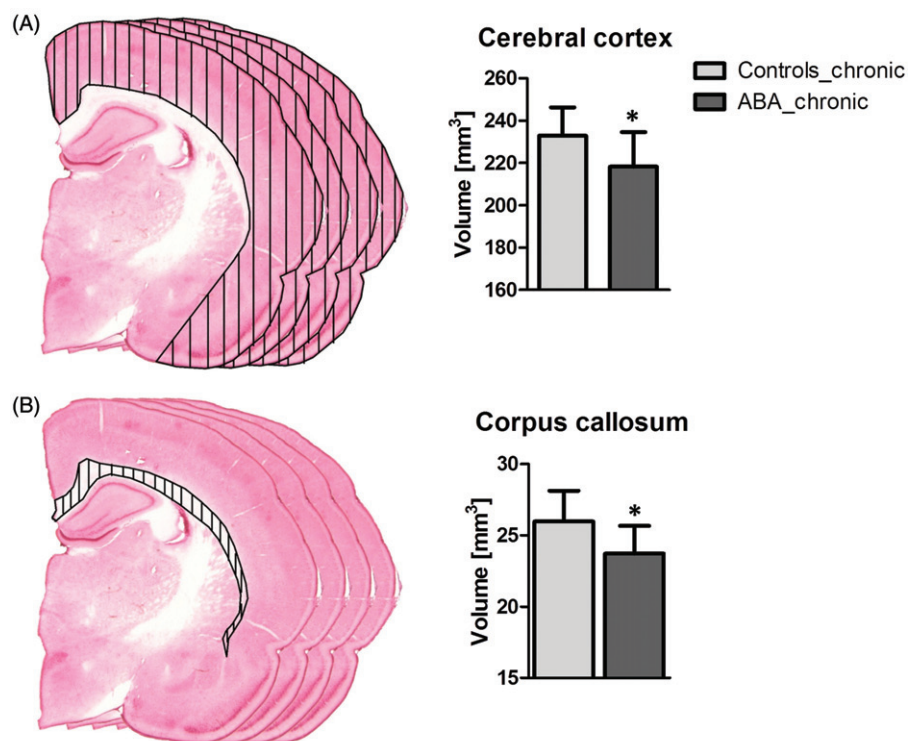


Figure 2. Effect of ABA chronic starvation on brain volume. The volumes (shaded) of (A) cerebral cortex and (B) corpus callosum were reduced in the ABA group ($n = 10$) compared to controls ($n = 12$). * $P \leq 0.05$, two-sided Student's t -test.

Grey matter and white matter volume

We previously demonstrated that the sizes of the cerebral cortex and white matter were significantly reduced in human patients with AN (Seitz et al. 2014, 2016). Similarly, a volume reduction of these brain areas was observed in ABA_chronic rats. This further validates the ABA model and underlines the importance of elucidating the underlying mechanism of this widespread volume reduction. Indeed, recent studies on large patient groups have shown that semi-starvation in AN seems to reduce grey and white matter volume in a global manner affecting most areas in the brain, with white matter deficits being especially predictive for clinical prognosis (King et al. 2015; Seitz et al. 2015).

Cellular changes in the ABA model

The cell numbers and surface area of GFAP-positive astrocytes were decreased in the cerebral cortex and corpus callosum of ABA rats, whereas no change in the number or area of neurons was found. As the ratio area/cell number was also significantly reduced (cerebral cortex, $P = 0.004$; corpus callosum, $P = 0.001$), each astrocyte appears to contain less GFAP protein per individual cell. Both results fit well with our finding of strongly reduced GFAP mRNA expression in ABA animals that show more than a 50% mRNA reduction

compared to controls. These findings suggest a strong influence of semi-starvation on astrocyte number, morphology and potentially function rather than favouring primary neuronal or oligodendroglial (myelination) changes after starvation. Earlier post-mortem human studies had suggested fine-grained changes in neuronal morphology (Martin 1958; Neumärker et al. 1997); however, they did not systematically examine glial cells. In the only study, focussing on glial cell numbers in AN, Reyes-Haro et al. (2015) found that the number of GFAP-positive astrocytes in the body of the corpus callosum (not in the splenium and genu) was significantly reduced in acute dehydration-induced anorexia rats but not in a food-restricted only group without dehydration. Furthermore, the astrocyte/glial cell ratio was lower in both starvation groups compared to controls. This is in line with our results. However, our findings are much more pronounced, potentially due to our longer duration of starvation in the chronic condition. Acute starvation did not suffice to produce similar effects in our study, underscoring the importance of illness duration, which has previously been implicated in the extent of brain volume loss in patients with AN (Boghi et al. 2011; Fonville et al. 2014). This astrocyte effect seems to be cell-type specific because the other major glial cell population, oligodendrocytes, was not affected. A potential mechanism for a marked reduction of astrocyte numbers

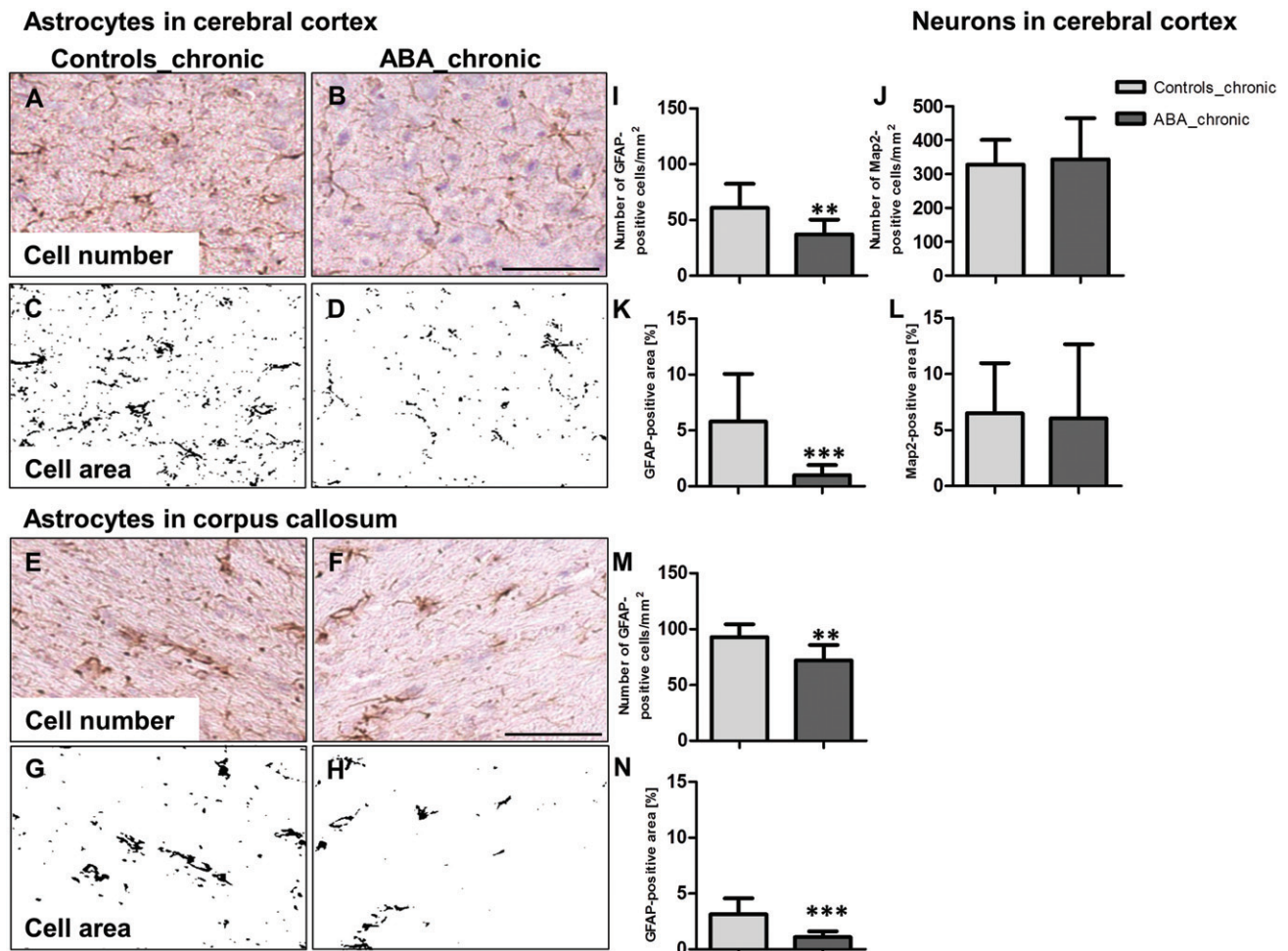


Figure 3. Cell number and cell area of GFAP-stained astrocytes in the (A–D) cerebral cortex and the (E–H) corpus callosum of ABA and control rats. Astrocyte cell number and cell areas were significantly reduced in both brain regions (I, K, M, N). The neuron cell number (J) and neuron cell area (L) were quantified with Map2, which showed no significant alteration in ABA rats compared to controls. Scale bar = 100 μ m. ** $P \leq 0.01$, *** $P \leq 0.001$, two-sided Student's *t*-test.

could be a reduction in cell neogenesis. This was shown by Barbarich-Marsteller et al. (2013) in an acute ABA model, where a significantly lower proliferation rate of new cells was found for glia in the hippocampus and corpus callosum but not for neurons.

Possible role of astrocytes in the starvation process

The brain volume reductions following starvation in AN were associated with a significant loss of GFAP-positive astrocyte numbers, reduction of their cell-size and overall GFAP gene expression levels. The latter being even more striking than the morphology, suggesting a functional down (de)-regulation of astroglia. GFAP is an intermediary filament of astrocytes thought to be responsible for the cell shape, the mechanical stability and communication of astrocytes with other astrocytes and neurons (Hol & Pekny 2015). Similar

reductions in glial cell number have been shown in the fronto-limbic areas of the brain, including the anterior cingulate and prefrontal cortices in patients with depression, which is very often comorbid in AN (Banasr et al. 2011; Verkhatsky et al. 2015). Their causal role is supported by animal studies in which rats displayed depressive symptoms after destruction of their frontocortical astrocytes (Rial et al. 2015). In both patient groups, a similar pathophysiological mechanism of astrocyte reduction could be at play. Also in major depressive disorder a reduction of GFAP has been shown, and impaired vesicle transport in astrocytes leads to memory deficits similar to those in AN (Elsayed & Magistretti 2015). Recently, astrocyte alterations were also found in other psychiatric diseases like anxiety and following chronic stress, partly linked to reduced GFAP (Elsayed & Magistretti 2015; Bender et al. 2016). This shows that astrocytes might play a much greater role in psychiatric

pathophysiology than previously thought (Stevens 2009). Astrocytes have multiple functions, such as constituting the blood–brain barrier, regulating neuronal activity, elimination of radical oxygen species and inflammation (Molofsky et al. 2012; Rose et al. 2013; Dringen et al. 2015; Kipp et al. 2016). One of the most

important roles of astrocytes is to supply energy to neurons, as neurons have little capacity to store energy themselves (Bélanger et al. 2011). Thus, fewer astrocytes containing less GFAP could further aggravate the energy metabolism of neurons in an already chronically energy-deprived state, leading to impairment of their proper functioning.

A growing body of literature shows that mature GFAP-positive astrocytes are interconnected in networks and can be indirectly and even directly involved in synaptic transmission, synapse formation and even large neural circuits, indicating a role in synaptic plasticity as well as learning and memory (Paixão & Klein 2010; Molofsky et al. 2012). Therefore, lower astrocyte numbers with fewer intermediary filaments may result in deficits in learning and memory (Henneberger et al. 2010). This could help explain the impairments in learning processes that were found to be associated with brain volume loss in patients with AN (Chui et al. 2008; Castro-Fornieles et al. 2010). In our previous studies, we could show that oestrogen deficiency in chronic ABA rats and patients with AN was associated with impaired memory function (Buehren et al. 2011; Paulukat et al. 2016). This association could be mediated by changed astrocyte function as astrocytes express all types of oestrogen receptors and are regulated by gonadal hormones (Garcia-Segura et al. 1999; Garcia-Segura & Melcangi 2006; Karki et al. 2014).

Our findings may be important regarding the difficulties of early psychotherapy treatment of patients with AN. As all therapeutic changes require learning and the adaption of new viewpoints, these processes could be disturbed due to a reduced number of astrocytes supporting synaptogenesis and long-term potentiation (Henneberger et al. 2010; Paulukat et al. 2016).

Limitations

To allow for standardised weight loss and the possibility to study chronic starvation effects, we slightly modified the original ABA experimental set-up

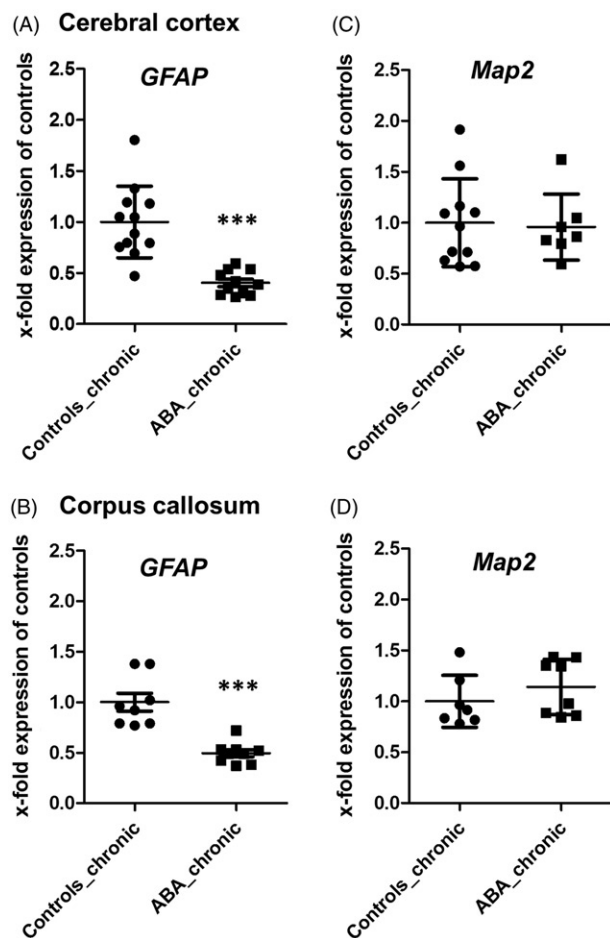


Figure 4. mRNA expression of GFAP in the (A) cerebral cortex and (B) corpus callosum, and mRNA expression of Map2 in the (C) cerebral cortex and (D) corpus callosum. In both brain regions, GFAP expression was downregulated in ABA rats compared to the control group, but there was no significant reduction in Map2 expression. *** $P \leq 0.001$, two-sided Student's t -test.

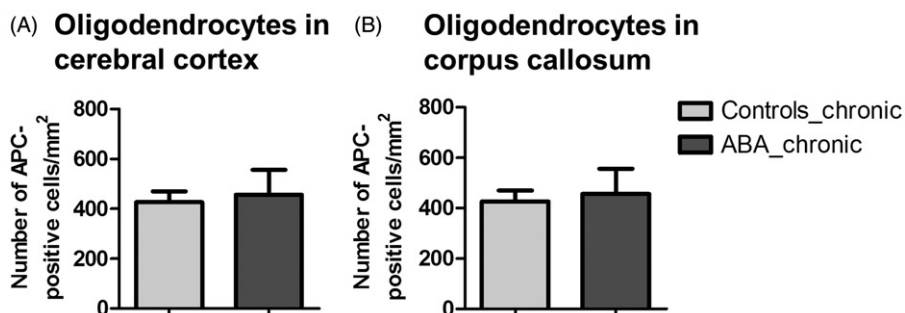


Figure 5. The number of APC-stained oligodendrocytes in the (A) cerebral cortex and (B) corpus callosum were not altered.

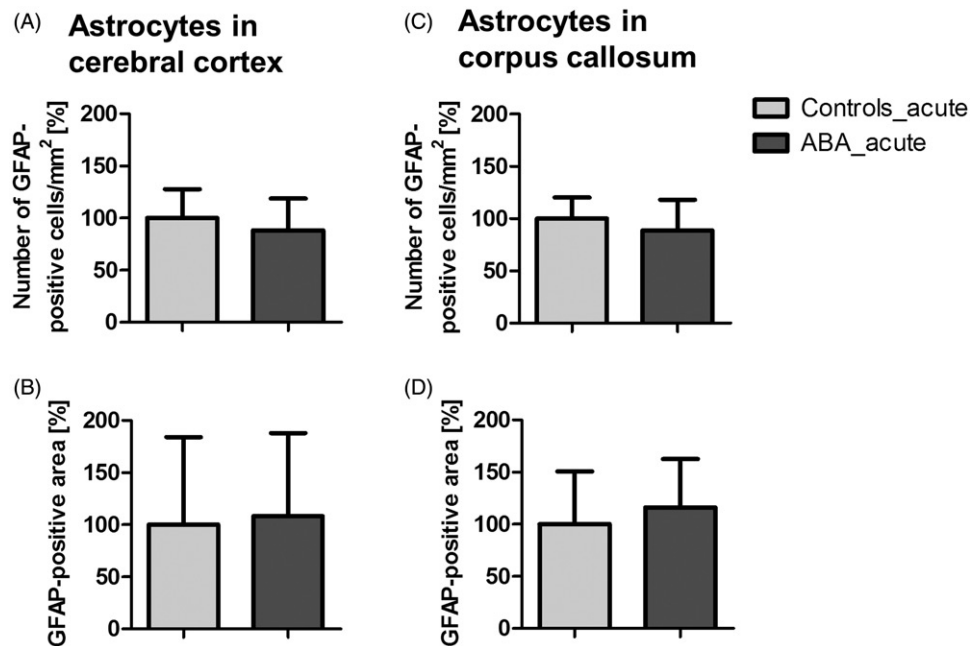


Figure 6. Controlling for duration of starvation. The cell number and cell area of GFAP-positive astrocytes of ABA_acute rats ($n=9$) in the (A, B) cerebral cortex and (C, D) corpus callosum were not significantly modified compared to Controls_acute ($n=9$) rats.

including a fixed weight loss, a weight-holding phase and omitting self-starvation (Paulukat et al. 2016). The original ABA set-up often proved to be lethal to the animals when continued for a longer time frame than a few days (Routtenberg & Kuznesof 1967; Exner et al. 2000).

GFAP is a marker for differentiated astrocytes, but it is not an absolute marker of all non-reactive astrocytes under healthy conditions (Olude et al. 2015), e.g., it labels the protoplasmic astrocytes in the cortex only poorly (Molofsky et al. 2012). Therefore, we might not have been able to detect the total number of astrocyte reduction in the cerebral cortex and corpus callosum in our study. Lastly, our study does not prove a causal role for astrocyte reduction in brain volume loss; e.g., both could be caused by an independent third factor. Further intervention studies including astrocyte manipulations are required to confirm causality.

Conclusion

We showed that volume reductions of the cerebral cortex and corpus callosum are observed in a chronic ABA model, which is in line with clinical findings in AN patients. The number of GFAP-positive cells and their immunoreactive surface area was strongly reduced in our rat model, which may explain the lower volumes in these regions. A changed functionality of astrocytes might thus represent an important consequence of starvation and play an important role in the

underlying pathobiology in AN, including metabolic processes, neuronal functioning and synapse formation. Future AN research should start to focus on GFAP-positive astrocytes in addition to pure neuronal functioning to establish potential links to deficits in learning and memory, slow psychotherapeutic change and depression in AN. Interventions targeting the functions and regeneration of astrocytes could open up a whole new treatment approach to address these core deficits in patients with AN and potentially also help patients with other causes of semi-starvation.

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